

PATENT

Attorney Docket No. 03495.0008-08000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Marc ALIZON et al.)
Application No.: 08/384,248) Group Art Unit: 1648
Filed: February 6, 1995) Examiner: J. Parkin
For: METHOD FOR DETECTING HUMAN) Confirmation No.: 9162
IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1))
REVERSE TRANSCRIPTASE (RT) ACTIVITY)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CLAIM FOR PRIORITY

Sir:

Under the provisions of Section 119(b) of 35 U.S.C., Applicant hereby claims the benefit of the filing date of Great Britain Patent Application Number 83 24800, filed September 15, 1983, for the above identified United States Patent Application.

A certified copy of GB 83 24800 was filed in grandparent application Serial No. 06/558,109 on January 25, 1984. A copy of this submission is attached as Exhibit 1.

A fee of \$1,410.00 is enclosed. Please charge any additional fees required in connection with the filing for this claim for priority to Deposit Account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: December 23, 2008

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of :
MONTAGNIER ET AL : Group Art Unit
Serial No. 558,109 : Examiner:
Filed: December 5, 1983 : 703-557-
For a Patent for : January 25, 1984
ANTIGENS,...DIAGNOSIS OF LYMPHA :
DENOPATHY etc. :

CLAIM OF PRIORITY

Hon. Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

In accordance with 35 USC 119, applicants hereby claim the benefit
of the filing date of their prior foreign patent application, namely:

Great Britain Patent Application No. 83 24800

Filed: September 15, 1983.

In support of this claim, a certified copy of the aforementioned
patent application is submitted herewith.

Respectfully submitted,

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I, the undersigned, being an officer duly authorised in accordance with Section 62(3) of the Patents and Designs Act 1907, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

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9th day of DECEMBER 1983

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PATENTS ACT 1977

15 SEPT 1983

PATENTS FORM No. 1/77 (Revised 1982)

(Rules 16, 19)

The Comptroller
 The Patent Office
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16/09/83 C0316 PAT*** 10.00

Fee: £10.00 1983
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REQUEST FOR GRANT OF A PATENT

83341800

THE GRANT OF A PATENT IS REQUESTED BY THE UNDERSIGNED ON THE BASIS OF THE PRESENT APPLICATION

I Agent's Reference KDG/25921

II Title of Invention Antigens, means and method for the diagnosis of lymphadenopathy and acquired immune deficiency syndrome

III Applicant or Applicants (See note 2)
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Country State

Address

IV Inventor (see note 3)

(a) The applicant(s) is/are the sole/joint inventor(s)
 or
 (b) A statement on Patents Form No. 7/77 if/ will be furnished

V Name of Agent (if any) (See note 4)

Reddie & Grose

ADP CODE NO

VI Address for Service (See note 5)

16 Theobalds Road
London WC1X 8PL

VII Declaration of Priority (See note 6)

Filing date

File number

Country _____

VIII The Application claims an earlier date under Section 8(3), 12(6), 15(4), or 37(4) (See note 7)

Section No. _____

Earlier application or patent number _____ and filing date _____

IX Check List (To be filled in by applicant or agent)

A The application contains the following number of sheet(s)

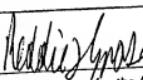
1 Request 1 Sheet(s)
2 Description 22 Sheet(s)
3 Claim(s) 2 Sheet(s)
4 Drawing(s) 1 Sheet(s)
5 Abstract 1 Sheet(s)

B The application as filed is accompanied by:-

1 Priority document NO
2 Translation of priority document NO
3 Request for Search NO
4 Statement of Inventorship and Right to Apply NO
5

X It is suggested that Figure No ... 1 of the drawings (if any) should accompany the abstract when published.

XI Signature (See note 8)


Reddie & Grose, Agents for the Applicant(s)

NOTES:

1. This form, when completed, should be brought or sent to the Patent Office together with the prescribed fee and two copies of the description of the invention, and of any drawings.
2. Enter the name and address of each applicant. Names of individuals should be indicated in full and the surname or family name should be underlined. The names of all partners in a firm must be given in full. Bodies corporate should be designated by their corporate name and the country of incorporation and, where appropriate, the state of incorporation within that country should be entered where provided. Full corporate details, e.g. "a corporation organised and existing under the laws of the State of Delaware, United States of America," trading styles, e.g. "trading as xyz company", nationality, and former names, e.g. "formerly [known as] ABC Ltd.", are not required and should not be given. Also enter applicant(s) ADP Code No. (if known).
3. Where the applicant or applicants is/are the sole Inventor or the joint inventors, the declaration (a) to that effect at IV should be completed, and the alternative statement (b) deleted. If, however, this is not the case the declaration (a) should be struck out and a statement will then be required to be filed upon Patent Form No 7/77.
4. If the applicant has appointed an agent to act on his behalf, the agent's name and the address of his place of business should be indicated in the spaces available at V and VI. Also insert agent's ADP Code No. (if known) in the box provided.
5. An address for service in the United Kingdom to which all documents may be sent must be stated at VI. It is recommended that a telephone number be provided if an agent is not appointed.
6. The declaration of priority at VII should state the date of the previous filing and the country in which it was made and indicate the file number, if available.
7. When an application is made by virtue of section 8(3), 12(6), 15(4), or 37(4) the appropriate section should be identified at VIII and the number of the earlier application or any patent granted thereon identified.
8. Attention is directed to rules 90 and 106 of the Patent Rules 1982.
9. Attention of applicants is drawn to the desirability of avoiding publication of inventions relating to any article, material or device intended or adapted for use in war (Official Secrets Acts, 1911 and 1920). In addition after an application for a patent has been filed at the Patent Office the comptroller will consider whether publication or communication of the invention should be prohibited or restricted under section 22 of the Act and will inform the applicant if such prohibition is necessary.
10. Applicants resident in the United Kingdom are also reminded that, under the provisions of section 23 applications may not be filed abroad without written permission or unless an application has been filed not less than six weeks previously in the United Kingdom for a patent for the same invention and no direction prohibiting publication or communication has been given or any such direction has been received.

DRAFTED

Antigens, means and method for the diagnosis of lymphadenopathy . . . and acquired immune deficiency syndrome..

The invention relates to antigens, means and methods for the diagnosis of lymphadenopathy and acquired immune deficiency syndrome.

The acquired immune deficiency syndrome (AIDS) 5 has recently been recognized in several countries. The disease has been reported mainly in homosexual males with multiple partners, and epidemiological studies suggest horizontal transmission by sexual routes as well as by intravenous drug administration, and blood transfusion.

10 The pronounced depression of cellular immunity that occurs in patients with AIDS and the quantitative modifications of subpopulations of their T lymphocytes suggest that T cells or a subset of T cells might be a preferential target for the putative infectious agent. Alternatively, these 15 modifications may result from subsequent infections. The depressed cellular immunity may result in serious opportunistic infections in AIDS patients, many of whom develop Kaposi's sarcoma. However, a picture of persistent multiple lymphadenopathies has also been described in homosexual 20 males and infants who may or may not develop AIDS. The histological aspect of such lymph nodes is that of reactive hyperplasia. Such cases may correspond to an early or a milder form of the disease.

It has been found that one of the major etiological 25 agents of AIDS and of lymphadenopathy syndrome (LAS), which is often considered as a prodromic sign of AIDS, should consist of a T-lymphotropic retrovirus which has been isolated from a lymph node of a homosexual patient with multiple lymphadenopathies. The virus appears to be distinct from the human

T-cell leukemia virus (HTLV) family (R.C. Gallo and H. S. Reitz, "J. Natl. Cancer Inst.", 69 (No. 6), 1209 (1982)). The last mentioned virus has been known as belonging to 5 the so-called HTLV-1 subgroup.

The patient was a 33-year-old homosexual male who sought medical consultation in December 1982 for cervical lymphadenopathy and asthenia (patient 1). Examination showed axillary and inguinal lymphadenopathies. Neither fever 10 nor recent loss of weight were noted. The patient had a history of several episodes of gonorrhea and had been treated for syphilis in September 1982. During interviews he indicated that he had had more than 50 sexual partners per year and had travelled to many countries, including 15 North Africa, Greece, and India. His last trip to New York was in 1979.

Laboratory tests indicated positive serology (immunoglobulin G) for cytomegalovirus (CMV) and Epstein-Barr virus. Herpes simplex virus was detected in cells 20 from his throat that were cultured on human and monkey cells. A biopsy of a cervical lymph node was performed. One sample served for histological examination, which revealed follicular hyperplasia without change of the general structure of the lymph node. Immunohistological 25 studies revealed, in paracortical areas, numerous T lymphocytes (OKT3⁺). Typing of the whole cellular suspension indicated that 62 percent of the cells were T lymphocytes (OKT3⁺), 44 percent were T-helper cells (OKT4⁺), and 16 percent were suppressor cells (OKT8⁺). 30 Cells of the same biopsied lymph node were put in culture medium, with phytohemagglutinin (PHA), T-cell growth factor (TCGF), and antiserum to human α interferon. The cells were grown in RPMI-1640 medium supplemented with antibiotics, 10^{-5} M β -mercaptoethanol, 10 percent fetal calf serum, 0.1 percent sheep antibody to human α interferon (neutralizing titer, 7 IU at 10^{-5} dilution and 10 percent TCGF, free of PHA. The reason for using the antiserum 35

to α -interferon was to neutralize endogenous interferon which is secreted by cells chronically infected by viruses, including retroviruses. In the mouse system, it had previously been shown that anti-serum to interferon could increase retrovirus production by a factor of 10 to 50-. F. Barré-Sinoussi et al., "Ann. Microbiol. (Institut Pasteur)" 130B, 349 (1979). After 3 days, the culture was continued in the same medium without PHA. Samples were regularly taken for reverse transcriptase assay and for examination in the electron microscope.

After 15 days of culture, a reverse transcriptase activity was detected in the culture supernatant by using the ionic conditions described for HTLV-I (B.J. Poiesz et al. "Proc. Natl. Acad. Sci. U.S.A." 77, 7415 (1980)). Virus production continued for 15 days and decreased thereafter, in parallel with the decline of lymphocyte proliferation. Peripheral blood lymphocytes cultured in the same way were consistently negative for reverse transcriptase activity, even after 6 weeks. Cytomegalovirus could be detected, upon prolonged co-cultivation with MRC5 cells, in the original biopsy tissue, but not in the cultured T lymphocytes at any time during culture.

The invention relates to the newly isolated virus 25 as a source of the above said antigen which will be defined later.

The newly isolated virus, which will hereafter be termed as LAV₁, will however be described first.

The virus is transmissible to cultures of T 30 lymphocytes obtained from healthy donors. Particularly virus transmission was attempted with the use of a culture of T lymphocytes established from an adult healthy donor of the Blood Transfusion Center at the Pasteur Institute. On day 3, half of the culture was co-

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cultivated with lymphocytes from the biopsy after centrifugation of the mixed cell suspensions. Reverse transcriptase activity could be detected in the supernatant on day 15 of the coculture but was not detectable on 5 days 5 and 10. The reverse transcriptase had the same characteristics as that released by the patient's cells and the amount released remained stable for 15 to 20 days. Cells of the uninfected culture of the donor lymphocytes did not exhibit reverse transcriptase activity 10 during this period or up to 6 weeks when the culture was discontinued.

The cell-free supernatant of the infected coculture was used to infect 3-day-old cultures of T lymphocytes from two umbilical cords, LC1 and LC5, in the 15 presence of Polybrene (2 μ g/ml). After a lag period of 7 days, a relatively high titer of reverse transcriptase activity was detected in the supernatant of both cord lymphocyte cultures. Identical cultures, which had not been infected, remained negative. These two successive infections 20 clearly show that the virus could be propagated on normal lymphocytes from either new-borns or adults.

In the above co-cultures one used either the cells of patient 1 as such (they declined and no longer grew) or cells which had been pre-X-rayed or mitomycin C-treated.

25 The LAV, virus, or LAV, virus suspensions, which can be obtained from infected cultures of lymphocytes have characteristics which distinguish them completely from other HTLV. These characteristics will be referred to hereafter and, when appropriate, in relation to the 30 Fig. 1 - this shows curves representative of variation of reverse transcriptase activity and [³H]uridine activity respectively versus successive fractions of the LAV, virus in the sucrose gradient, after ultracentrifugation therein of the virus contents of a cell-free 35 supernatant obtained from a culture of infected lymphocytes.

The analysis of LAV₁ virus by resorting to reverse transcriptase activity can be carried out according to the procedure which was used in relation to virus from patient 1. The results of the analysis are illustrated in Fig. 1. Cord blood T lymphocytes infected with virus from patient 1 were labelled for 18 hours with [³H]uridine (28 Ci/mmol, Amersham; 20 μ Ci/ml). Cell-free supernatant was ultracentrifuged for 1 hour at 50,000 rev/min. The pellet was resuspended in 200 μ l of NTE buffer (10 mM tris, pH 7.4, 100 mM NaCl, and 1 mM EDTA) and was centrifuged over a 3-ml linear sucrose gradient (10 to 60 percent) at 55,000 rev/min for 90 minutes in an IEC type SB 498 rotor. Fractions (200 μ l) were collected, and 30 μ l samples of each fraction were assayed for DNA RNA dependant polymerase activity with 5 mM Mg²⁺ and poly(A)-oligo-(dT)₁₂₋₁₈ as template primer; a 20- μ l portion of each fraction was precipitated with 10 percent trichloroacetic acid and then filtered on a 0.45- μ m Millipore filter. The ³H-labelled acid precipitable material was measured in a Packard β -counter.

That the new virus isolate was a retrovirus was further indicated by its density in the above sucrose gradient, which was 1.16, and by its labelling with [³H]uridine (fig. 1). A fast sedimenting RNA appears to be associated with the LAV₁ virus.

Virus-infected cells from the original biopsy as well as infected lymphocytes from the first and second viral passages were used to determine the optimal requirements for reverse transcriptase activity and the template specificity of the enzyme. The results were the same in all instances. The reverse transcriptase activity displayed a strong affinity for poly(adenylate-oligodeoxythymidylate)[poly(A)-oligo(dT)₁₂₋₁₈], and required Mg²⁺ with

an optimal concentration (5mM) and an optimal pH of 7.6. The reaction was not inhibited by actinomycin D. This character, as well as the preferential specificity for ribosadenylate-deoxythymidylate over deoxyadenylate-
5 deoxythymidylate, distinguish the viral enzyme from DNA-dependent polymerases.

Electron microscopy of ultrathin sections of virus-producing cells shows two types of particles, presumably corresponding to the immature and mature 10 forms of the virus : immature particles are budding at the cell surface, with a dense crescent in close contact with the plasma membrane. Occasionally, some particles remain in this state, while being freed from the cell surface.

15 Mature particles have a quite different morphology with a small, dense, eccentric core (mean diameter: 41 nm). Most virions are round (mean diameter: 139 nm) or ovoid, but in some pictures, especially in the particles seen in the original culture from which 20 the virus was isolated, a tailed morphology can also be observed. The latter form can also be observed in cytoplasmic vesicles which were released in the medium. Such particles are also formed by budding from vesicle membranes.

25 Morphology of mature particles is clearly distinct from HTLV, whose large core has a mean diameter of 92 nm.

30 Helper T-lymphocytes (Leu 3 cells) form the main target of the virus. In other words the LAV, virus has particular tropism for these cells. Leu 3 cells are recognizable by the monoclonal antibodies commercialized by ORTHO under the trademark OKT4. In contrast enriched cultures of Leu 2 cells, which are mainly suppressor or 35 cytotoxic cells and which are recognized by the monoclonal antibodies commercialized by ORTHO under the

trademark OKT8 did not produce, when infected under the same conditions, any detectable RT activity even 6 weeks after virus infection.

In most cases of AIDS, the ratio of OKT4⁺ over 5 OKT8⁺ cells which is normally over 1, is depressed to values as low as 0.1 or less.

The LAV₁ virus is also immunologically distinct from previously known HTLV-1 isolates from cultured T lymphocytes of patients with T lymphomas and T leu-10 kemias. The antibodies used were specific for the p19 and p24 core proteins of HTLV-1. A monoclonal anti-body to p19 (M. Robert-Guroff et al. "J. Exp. Med." 154, 1957 (1981)) and a polyclonal goat antibody to p24 (V.S. Kalyanaraman et al. "J. Virol.", 38, 906 (1981)) 15 were used in an indirect fluorescence assay against infected cells from the biopsy of patient 1 and lymphocytes obtained from a healthy donor and infected with the same virus. The LAV₁ virus-producing cells did not react with either type of antibody, whereas two lines of 15 cord lymphocytes chronically infected with HTLV-1 (M. Popovic, P.S. Sarin, M. Robert-Guroff, V. S. Kalyanaraman, D. Mann, J. Minowada, R.C. Gallo, "Science" 219, 856 (1983)) and used as controls showed strong surface fluorescence.

20 In order to determine which viral antigen was recognized by antibodies present in the patient's sera, several immunoprecipitation experiments were carried out. Cord lymphocytes infected with virus from patient 1 and uninfected controls were labelled with [³⁵S]methionine 25 for 20 hours. Cells were lysed with detergents, and a cytoplasmic S10 extract was made. Labelled virus released in the supernatant was banded in a sucrose gradient. Both materials were immunoprecipitated by antiserum to HTLV-1 p24, by serum from patient 1, and by serum 30 samples from healthy donors. Immunocomplexes were

analyzed by polyacrylamide gel electrophoresis under denaturing conditions. A p25 protein present in the virus-infected cells from patient 1 and in LCL cells infected with this virus, was specifically recognized by serum from patient 1 but not by antiserum to HTLV-1 p24 obtained under similar conditions or serum of normal donors. Conversely the p24 present in control HTLV-infected cell extracts was recognized by antibodies to HTLV but not by serum from patient 1.

10 The main protein (p25) detected after purification of ^{35}S -methionine-labelled virus has a molecular weight of about 25,000 (or 25K). This is the only protein recognized by the serum of patient 1. By analogy with other retroviruses, this major protein was considered 15 to be located in the viral core.

This can be confirmed in immuno-electron microscopy experiments, which show that the patient's serum can agglutinate the viral cores. Conversely, an antiserum raised in rabbit against an other retrovirus did not 20 precipitate the p25 protein.

The viral origin of other proteins seen in polyacrylamide gel electrophoresis of purified virus is more difficult to assess. A p15 protein could be seen after 25 silver staining, but was much weaker after ^{35}S -methionine perhaps due to the paucity of this amino-acid in the protein. In the higher MW range, a contamination of the virus by cellular proteins, either inside or outside the viral envelope, is likely. A 36K and a 42K protein and a 60K protein were constantly formed to be associated 30 with the purified virus and may represent the major envelope proteins.

No p 19 (having a molecular weight of about 19 mM) was isolated from LAV extracts.

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The invention concerns more particularly the extracts of said virus as soon as they can be recognized immunologically by sera of patients afflicted with LAS or AIDS. Needless say any type of immunological assay 5 may be brought into play. By way of example immunofluorescence or immunoenzymatic assays or radio-immunoprecipitation tests are particularly suitable.

As a matter of fact and except under exceptional circumstances, sera of diseased patients do not recognize 10 the intact LAV₁ virus, or viruses having similar phenotypical or immunological properties. The envelope proteins of the virus appeared as not detectable immunologically... by the patients' sera. However as soon as 15 the core proteins become exposed to said sera, the immunological detection becomes possible. Therefore the invention concerns all extracts of the virus, whether it be the crudest ones - particularly mere virus lysates - or the more purified ones, particularly extracts enriched in the p25 protein or even the purified p25 protein or in protein immunologically related therewith. Any purification procedure may be resorted to. 20 By way of example only, one may use purification procedures such as disclosed by R.C. Montelaro et al, J. of Virology, June 1982, pp. 1029-1038.

The invention concerns more generally extracts 25 of any virus having similar phenotype and immunologically related to that obtained from LAV₁. Sources of viruses of the LAV type consist of T-lymphocyte cultures isolatable from LAS- and AIDS - patients or from haemophiliacs.

In that respect other preferred extracts are those obtained from two retroviruses obtained by propagation on normal lymphocytes of the retroviruses isolated from :

5 1) lymph node lymphocytes of a caucasian homosexual with multiple partners, having extensive Kaposi sarcoma lesions and severe lymphopenia with practically no OKT4⁺ lymphocytes in his blood ;
2) blood lymphocytes of a young B haemophiliac
10 presenting neurotoxoplasmosis and OKT4⁺/OKT8⁺ ratio of 0.1.

These two retroviruses have been named IDAV1 and IDAV2 respectively (for Immune Deficiency Associated Virus). Results of partial characterization obtained so far indicate similarity - if not identity - of IDAV1 and IDAV2 to LAV1 :

- same ionic requirements and template specificities of reverse transcriptase,
- same morphology in ultrathin sections,
- 20 - antigenically related p25 proteins : serum of LAV1 patient immunoprecipitates p25 from IDAV1 and IDAV2 ; conversely, serum from IDAV2 patient immunoprecipitates LAV1:p25.

IDAV1 patient serum seemed to have a lower anti-bodies titer and gave a weak precipitation band for LAV1 and IDAV1 p25 proteins. The p25 protein of IDAV1 and IDAV2 was not recognized by HTLV p24 antiserum.

These similarities suggest that all these three isolates belong to the same group of viruses.

30 The invention further relates to a method of in vitro diagnosis of LAS or AIDS, which comprises contacting a serum or other biological medium from a patient to be diagnosed -----

with a virus extract as above defined and detecting the immunological reaction.

Preferred methods bring into play immunoenzymatic or immunofluorescent assays, particularly according to the ELISA technique. Assays may be either direct or indirect immunoenzymatic or immunofluorescent assays.

Thus the invention also relates to labelled virus extracts whatever the type of labelling : enzymatic, fluorescent, radioactive, etc..

10 Such assays include for instance :

- depositing determined amounts of the extract according to the invention in the wells of titration microplate;
- introducing in said wells increasing dilutions of the serum to be diagnosed;
- 15 - incubating the microplate;
- washing the microplate extensively;
- introducing in the wells of the microplate labelled antibodies directed against blood immunoglobulins, the labelling being by an enzyme
- 20 selected from those which are capable of hydrolysing a substrate, whereby the latter then undergoes a modification of its absorption of radiations, at least in a determined wavelength band and
- detecting, preferably in a comparative manner with
- 25 respect to a control, the amount of substrate hydrolysis as a measure of the potential risk or effective presence of the disease.

The invention also relates to kits for the above-said diagnosis which comprise :

- 30 - an extract or more purified fraction of the abovesaid types of viruses, said extract or fraction being labelled, such as by a radioactive, enzymatic or immunofluorescent label ;

- human anti-immunoglobulins or protein A (advantageously fixed on a water-insoluble support such as agarose beads) ;
- a lymphocyte extract obtained from a healthy person ;
- 5 - buffers and, if appropriate, substrates for the visualization of the label.

Other features of the invention will further appear as the description proceeds of preferred isolation and culturing procedures of the relevant virus, of 10 preferred extraction methods of an extract suitable as diagnostic means, of a preferred diagnosis technique and of the results that can be achieved.

1. VIRUS PROPAGATION :

- 15 Cultured T-lymphocytes from either umbilical cord or blood or bone marrow cells from healthy, virus negative, adult donors are suitable for virus propagation.

There is however some variation from individual 20 to individual in the capacity of lymphocytes to grow the virus. Therefore, it is preferable to select an adult healthy donor, having no antibodies against the virus and whose lymphocytes repeatedly did not release spontaneously virus, as detected by reverse transcriptase 25 activity (RT) nor expressed viral proteins.

Lymphocytes of the donor were obtained and separated by cytophoresis and stored frozen at -180°C in liquid nitrogen, in RPMI 1640 medium, supplemented with 50 % decomplemented human serum and 10 % DMSO.

- 30 For viral infection, lymphocytes were put in culture (RPMI 1640) medium, with phytohaemagglutinin (PHA) at the concentration of 5.10^6 cells/ml for 3 days. Then, the medium was removed and cells resuspended in viral suspension (crude supernatant of virus-

producing lymphocytes, stored at -80°C). Optimal conditions of cell/virus concentrations were 2.10^6 cells for 5 to 10,000 cpm of RT activity, the latter determined as previously described. After 24 hours, cells were 5 centrifuged to remove the unadsorbed virus and resuspended in culture PHA-free medium and supplemented with PHA-free TCGF (Interleukin 2) : (0.5 - 1 U/ml, final concentration), POLYBREN (Sigma) 2 μ g/ml and anti-interferon α sheep serum, inactivated at 56°C for 30 minutes (0.1 % of a 10 serum which is able to neutralize 7 U of α leucocyte interferon at a 1/100,000 dilution).

Virus production was tested every 3 days by RT activity determination of 1 ml samples.

The presence of anti-interferon serum is important 15 in virus production : when lymphocytes were infected in the absence of anti-human- α -interferon serum, virus production, as assayed by RT activity, was very low or delayed. Since the sheep antiserum used was raised against partly purified α leucocyte interferon, made according to 20 the Cantell technique, the role of components other than interferon cannot be excluded.

Virus production starts usually from day 9 to 15 after infection, and lasts for 10-15 days. In no cases was the emergence of a continuous permanent line observed.

25 2. VIRUS PURIFICATION :

For its use in ELISA, the virus was concentrated by 10 % polyethyleneglycol (PEG 6000) precipitation and banded twice to equilibrium in a 20-60 % sucrose gradient. The viral band at density 1.16 is then recovered and 30 usable as such for ELISA assays.

For use in RIPA (radio-immune precipitation assay), purification in isotonic gradients of Metrizamide (sold under the trademark NYCODENZ by Nyegaard, Oslo) was found to be preferable. Viral 5 density in such gradients was very low (1.10-1.11).

Metabolic labelling with ^{35}S -methionine of cells and virus (RIPA) followed by polyacrylamide gel electrophoresis were performed as above described, except for the following modifications for RIPA: virus purified 10 NYCODENZ was lysed in 4 volumes of RIPA containing 500 U/ml of aprotinin. Incubation with 5 μl of serum to be tested was made for 1 hour at 37°C and then 18 hours at +4°C. Further incubation of the immunocomplexes with protein A SEPHAROSE beads was for 3 hours at +4°C.

15 3. PREPARATION OF THE VIRUS EXTRACT FOR ELISA ASSAYS :

Virus purified in sucrose gradient as above described, is lysed in RIPA buffer (0.5 % SDS) and coated on wells of microtest plates (Nunc).

Preferred conditions for the ELISA assay are:

20

After addition to duplicate wells of serial dilutions of each serum to be tested, the specifically fixed IgGs are revealed by goat anti-human IgG coupled with peroxidase. The enzymatic reaction is carried out on 25 ortho-phenylene-diamine as substrate and read with an automatic spectrophotometer at 492 nm.

On the same plate each serum is tested on a control antigen; a crude cytoplasmic lysate of uninfected T-lymphocytes from the same donor is used

in order to eliminate unspecific binding, which can be high with some sera.

Sera are considered as positive (antibodies against the virus) when the difference between O.D. against the viral antigen and O.D. against control cellular antigen is at least 0.30.

Hereafter there is disclosed a specific test for assaying the above mentioned disease or exposure to disease risks.

10 Method.

This ELISA test is for detecting and titration of seric anti-retrovirus type LAV antibodies.

It comprises carrying out a competition test between a viral antigen (cultivated on T lymphocytes) and 15 a control antigen constituted by a lysate of the same though non-infected lymphocytes.

The binding of the antibodies on the two antigens is revealed by the use of a human antoglobulin labelled with an enzyme which itself is revealed by 20 the addition of a corresponding substrate.

Preparation of the viral antigen.

The cellular cultures which are used are T lymphocytes of human origin which come from :

- 25 . umbilical cord, blood,
- . bone marrow,
- . blood of a healthy donor.

After infection of the cells by the virus, the supernatant of the infected cell culture is used. It is concentrated by precipitating with 10 % PEG, then 30 purified (two or three times) on a (20-60 %) sucrose gradient by ultracentrifugation to equilibrium.

The viral fractions are gathered and concentrated by centrifugation at 50 000 rotations per minute for 60 minutes.

The sedimented virus is taken in a minimum volume of buffer MTE at pH 7.4 (Tris 0.01 M, NaCl 0.1 M, EDTA 0.001 M).

5 The proteic concentration is determined by the Lowry method.

The virus is then lysed by a (RIPA + SDS) buffer (0.5 % final) for 15 minutes at 37°C.

Preparation of the control antigen.

10 The non-infected lymphocytes are cultured according to the preceding conditions for from 5 to 10 days. They are centrifuged at low speed and lysed in the RIPA buffer in the presence of 5 % of the product commercialized under the name of ZYMOFREN (Spécial) (500 u/ml). After 15 minutes at 4°C with 15 frequent stirrings with vortex, the lysate is centrifuged at 10 000 rotations per minute. The supernatant constitutes the control antigen. Its concentration in protein is measured by the Lowry method.

Reagents.

20 1 - Plates : NUNC - special controlled ELISA
 2 - Buffer PBS : pH 7.5
 3 - TWEEN 20
 4 - Carbonate buffer : pH = 9.6 ($CO_3^{2-}Na_2 = 0.2\text{ M}$
 $(CO_3^{2-}Na = 0.2\text{ M}$)
 25
 5 - Non foetal calf serum : which is stored in frozen state (BIOPRO),
 6 - Bovine serum albumin (BSA) SIGMA (fraction V)
 7 - Human anti IgG (H+L) labelled with peroxidase
 30 PASTEUR, in tubes of 1 ml preserved at 4°C
 8 - Washing buffer = PBS buffer, pH 7.5 + 0.05 % TWEEN 20
 Dilution of the conjuguate is carried out at the dilution indicated in PBS buffer + TWEEN 20 (0.05%) + bovine albumin 0.5 g per 100 ml
 35

9 - Dilution buffer of sera = PBS buffer + 0.05 % TWEEN 20

+ 0.5 g BSA

bovine serum albumin per
100 ml

5 10 - Substrate = OPD

. Citrate buffer pH = 5.6 trisodic citrate

($C_6H_5Na_3O_7$, $2H_2O$), 0.05 M ; citric acid
($C_6H_8O_7$, $1H_2O$), 0.05 M.

10 . Hydrogen peroxide = at 30 % (110 volumes) - used
at 0.03 % when using citrate buffer.

. Orthophenylene diamine = SIGMA

75 mg per 25 ml of buffer - which is diluted in
buffer extemporaneously.

Preparation of the plates.

15 The plates which are used have 96 U-shaped wells
(NUNC: ELISA). They include 12 rows of 8 wells each,
numbered from 1 to 12.

The distribution of antigens is as follows :

20 - 100 μ l of the viral antigen, diluted in carbonate
buffer at pH 9.6, is deposited in each of the wells of
rows marked \odot

- 1 - 2 - 5 - 6 - 9 - 10

- 100 μ l of the control antigen, diluted in carbonate
buffer at pH 9.6, is deposited in each of the wells of
25 rows marked \odot

3 - 4 - 7 - 8 - 11 - 12.

The dilution of the viral antigen is titrated
at each viral production. Several dilutions of viral
30 antigen are tested and compared to positive and negative
known controls (at several dilutions) and to human
anti-IgG labelled with peroxidase, the latter being
also tested at several dilutions.

As a rule, the protein concentration of the
preparation is of 5 to 2.5 μ g/ml.

The same proteic concentration is used for the control antigen.

The plates are closed with a plastic lid and are incubated overnight at 4°C.

5 Then they are put once in distilled water and centrifuged. The wells are then filled with 300 μ l of non foetal calf serum at 20 % in PBS buffer.

The incubation lasts 2 hours at 37°C (covered plates).

10 The plates are washed 3 times in PBS buffer with TWEEN 20, 0.05 % (PBS-tw buffer) :

- . first washing 300 μ l
- . second and third washing 200 μ l/well.

15 The plates are carefully dried and sealed with an adhesive plastic film. They can be stored at -80°C.
ELISA reaction : antibody titer assay.

After defreezing, the plates are washed 3 times in PBS-TWEEN. They are carefully dried.

20 The positive and negative control sera as well as the tested sera are diluted first in the tube, with PBS-TWEEN containing 0.5 % bovine albumin.

The chosen dilution is 1/40.

- 100 μ l of each serum are deposited in duplicate on the viral antigen and in duplicate on the control antigen.

25 - The same is carried out for the positive and negative diluted sera.
- 100 μ l of PBS + TWEEN + bovine serum albumin are introduced in two wells \ominus and in two wells \ominus to form the conjugated controls.

30 The plates equipped with their lids are incubated for 1 h 30 at 37°C.

They are washed 4 times in PBS + TWEEN 0.05 %.

- 100 μ l of human anti-IgG (labelled with peroxidase) at the chosen dilution are deposited in each well and

35 incubated at 37°C.

The plates are again washed 5 times with the (PBS + TWEEN) buffer. They are carefully dried.

Revealing the enzymatic reaction is carried out by means of a orthophenylenediamine substrate (0.05 % 5 in citrate buffer pH 5.6 containing 0.03 % of H_2O_2).

100 μ l of substrate is distributed in each well.

The plates are left in a dark room for 20 minutes at the laboratory temperature.

Reading is carried out on a spectrophotometer 10 (for microplates) at 492 nm.

Sera deemed as containing antibodies against the virus are those which give a ODD (optical density difference = optical density of viral antigen less optical density of control antigen) equal to or higher than 0.30.

15 This technique enables a qualitative titration as well as a quantitative one. For this purpose, it is possible either to use several dilutions of the serum to be assayed, or to compare a dilution of the serum with a 20 range of controls tested under the same conditions.

The table hereafter provides first results of serological investigations for LAV antibodies, carried out by using the above exemplified ELISA assay.

FIRST RESULTS OF SEROLOGICAL INVESTIGATIONS FOR LAV
ANTIBODIES IN FRANCE

	Total examined	ELISA-LAV positive	%positive	ELISA-HTLV ¹ (Biotech) positive	%positive
Lymphadenopathy patients*	35	22	(63)	5 ^{***}	(14)
Healthy homosexuals	40	7	(17)	1	(3)
Control population	54	1	(1,9)	0	(<2,6)

* 28 homosexuals

3 Haitians (1 woman)

4 toxicomans (2 women)

** The number of positive sera is probably overestimated in this test, since no control of unspecific binding could be done.

*** Out of the 5 LAS HTLV¹ positive, 3 were born in Haiti, 1 had stayed for a long time in Haiti and 1 had made several travels to USA.
All of them had also antibodies against LAV.

The table shows clearly high prevalence of LAV antibodies in the homosexual patients with LAS, the very low incidence in the normal population and also a moderate spread of virus infection in still healthy homosexuals. In the latter group, all the positive individuals had a high number of partners (>50 per year). The incidence of HTLV antibodies was very low in all three groups (determined by using a commercial

ELISA test (Biotec)). The groups of AIDS patients gave less interpretable results : approximatively 20 % had LAV antibodies, but some of the sera were taken at a very late stage of the disease, with a possible negati-

5 vation of the humoral response.

It should further be mentioned that lymphocytes of LAS patients do not produce detectable amounts of LAV-type virus. Particularly cells of lymph nodes from 6 more LAS patients were put in culture and 10 tested for virus production, as described for patient 1. No virus release could be detected by RT activity. However, a p25 protein recognized by the serum of the first patient could be detected in cytoplasmic extracts of the T-cells labelled with 35 S-methionine in 3 other 20 cases. This suggests partial expression of a similar virus in such cases. Moreover, all (6/6) of these patients had antibodies against LAV p25 proteins, indicating that they all had been infected with a similar or identical virus.

25 Interestingly, in lymphocytes of one of the patients (patient 2), there was a weak but definite immunoprecipitation of a band of similar size (p24-p25) with goat antiserum raised against HTLV1. Similarly, the patient's serum had antibodies against both HTLV and 30 LAV, suggesting a double infection by either virus. Such cases seem rather infrequent.

The invention finally also relates to the biological reagents that can be formed by the LAV extracts containing the p25 protein or by the purified 35 p25 protein, particularly for the production of antibodies directed against p25 in animals or of monoclonal antibodies. These antibodies are liable to form useful tools in the further study of antigenic determinants of LAV viruses or LAV-related viruses.

It is acknowledged that the OKT designations which have been used with respect to the designation of some sub-sets of lymphocytes or related monoclonal antibodies, for ease of language, should in no way be opposed to the validity of any corresponding trademark, whether registered or not by its owner.

It should further be mentionned that the viral extracts, particularly viral lysates or enriched fractions can also be defined by reference to their immunological relationship or similitude with the extracts or enriched fractions containing a p25 protein as obtainable from the strain LAV1, IDAV1 or IDAV2. Thus any protein fraction which is capable of giving similar patterns of immunological reaction as do the protein extracts of LAV1, IDAV1 or IDAV2 with the same sera, must be considered as equivalent thereof and, accordingly, be deemed as encompassed by the present invention. A similar conclusion extends of course to the diagnostic means (process and kits) which may make use of such 2.0 equivalent protein extracts.

The LAV1 virus has been deposited at the "Collection Nationale des Cultures de Micro-organismes" (C.N.C.M.) under n° I-232 on July 15, 1983 and IDAV1 and 25 IDAV 2 viruses have been deposited at the C.N.C.M. on September 15, 1983 under n° I-240 and I-241, respectively. The invention encompasses as well the extracts of mutants or variants of the above deposited strains as long as they possess substantially the same immunological properties.

CLAIMS :

- 1 - Retrovirus extract immunologically recognized by sera of LAS and AIDS afflicted patients and obtained from a T-lymphotropic-retrovirus, whose preferential target consists of Leu 3 cells, which has reverse transcriptase activity requiring the presence of Mg^{2+} ions and displaying a strong affinity for poly(adenylate-oligodeoxy-thymidylase) [poly(A)-oligo(dT)₁₂₋₁₈], which has a density of 1.16 in a sucrose gradient, which has a mean diameter of 139 nanometre and a core of mean diameter of 41 nanometre, whose envelope proteins are not detected immunologically by the sera of LAS and AIDS afflicted patients, which is recognized immunologically by said sera and which contains a p25 protein which is not recognized immunologically by a p24 protein of HTLV virus, which is free of a p19 protein.
- 2 - Retrovirus extract immunologically recognized by sera of LAS and AIDS afflicted patients and obtainable from any of the retrovirus deposited at the C.N.C.M. under n° I-232, I-240 and I-241.
- 3 - A retroviral extract which is immunologically related to the retroviral extracts of claims 1 or 2.
- 4 - The retroviral extract of any of claims 1 to 3 which consists of the crude lysate of said retrovirus.
- 5 - The p25 protein of the extracts of any of claims 1 to 4 which is in the purified state.
- 6 - A method for the in vitro diagnosis of LAS or AIDS, which comprises contacting a serum or other biological medium obtained from the patient to be diagnosed with the retrovirus extract of any of claims 1 to 4 or with the purified p25 protein of claim 5 and detecting the immunological reaction.
- 7 - A kit for assay of sera from LAS or AIDS afflicted patients which comprises :
 - 1 - retroviral extract according to any of claims 1 to 4 or 35 the purified protein of claim 5, which are labelled ;

- human anti-immunoglobulins;
- a lymphocyte extract obtained from a healthy person ;
- buffers and, if appropriate, substrates for the
5 vizualisation of the label ;
- means to detect the labelled conjugate resulting from
the immunological reaction between the labelled reagent
and the assayed serum.

8 - A kit for AIDS or LAS assay which comprises :

- 10 - a retroviral extract according to any of claims 1 to 4
or the purified protein of claim 5 ;
- labelled human anti-immunoglobulins ;
- a lymphocyte extract obtained from a healthy person ;
- buffers and, if appropriate, substrate for the vizuali-
15 sation of the label ;
- means to detect the labelled conjugate resulting from the
immunological reaction between the labelled reagent and the
assayed serum.

Figure(s) to accompany abstract

ABSTRACT

Antigens, means and method for the diagnosis of lymphadenopathy
and acquired immune deficiency syndrome

The invention concerns a retrovirus extract containing a p25 protein which recognizes immunologically sera of patients afflicted with lymphadenopathy syndrom (LAS) or acquired immune deficiency syndrom (AIDS). It relates to a method and kit for in vivo assay of LAS or AIDS involving contacting sera from patients to be diagnosed for such diseases with said retrovirus extract and by detecting the immunological reaction, if any.

DECLARATION FOR PATENT APPLICATION (U.S.
(INCLUDING DESIGN PATENT APPLICATIONS)

Attorney's Docket No.

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the invention which is claimed and for which a patent is sought on the invention entitled "Antigens, means and method for the diagnosis of lymphadenopathy and _____ the specification of which is attached hereto was filed on 5-12-1983 asApplication Serial No. 558,109

and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign applications for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

83 24800

Great Britain

September 15, 1983

Priority Claimed

(Number)

(Country)

(Day/Month/Year Filed)

Yes No

(Number)

(Country)

(Day/Month/Year Filed)

Yes No

(Number)

(Country)

(Day/Month/Year Filed)

Yes No Additional applications identified on attached sheet

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

 Additional applications identified on attached sheet

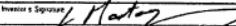
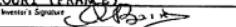
I hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issued thereon.

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DECLARATION FOR PATENT APPLICATION (US)
INCLUDING DESIGN PATENT APPLICATIONS

Attorney's Docket No

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name:

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **"Antigens, means and method for the diagnosis of lymphadenopathy and**, the specification of which

(check is attached hereto.
 one) was filed on 5.12.1983 as
 Application Serial No. 558,109
 and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

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Prior Foreign Application(s)

<u>.83 24800</u>	<u>Great Britain</u>	<u>September 15, 1983</u>	<u>Priority Claimed</u>
(Number)	(Country)	(Day/Month/Year Filed)	Yes <input type="checkbox"/> No <input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes <input type="checkbox"/> No <input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes <input type="checkbox"/> No <input type="checkbox"/>

 Additional applications identified on attached sheet

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

 Additional applications identified on attached sheetI hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:
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 I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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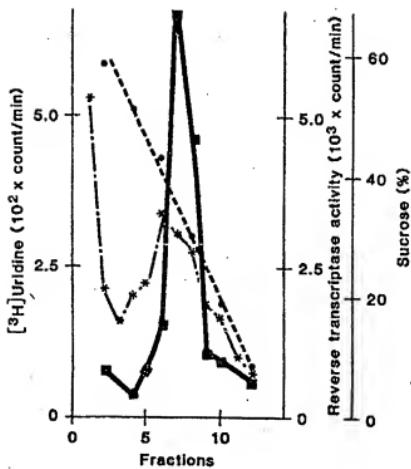


FIG 1

— measure of reverse transcriptase activity on successive fractions of sucrose gradient.

— · — measure of acidic precipitable material labelled with [3 H] uridine.

— · · — density variation of the gradient.

ALL INFORMATION CONTAINED